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13. ABSTRACT (Maximum 200 words)

The intent of this contract was to help create an x-ray microscopy facility at the Laser Plasma Laboratory at CREOL specially dedicated to applications to biology. The hope of this contract was that, in setting up a dedicated facility here in the US, we might attract the collaboration of medical and biological researchers, and thereby demonstrate the usefulness of this form of x-ray analysis. Hopefully then further development of this approach could then be supported by research funds from the medical and biological sciences.

We have been successful in this endeavor. We have established a facility for biological microscopy. This facility will soon be a dedicated facility to this activity. We have produced our own x-ray images of biological specimens. Most encouragingly, we have formed collaboration with several groups of biologists and medical scientists to exploit this technology further.

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Final Technical Report

Time-resolved, high-resolution, x-ray microscopy of *in-vitro* biological and life science specimens with the aid of laser plasmas.

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Contract F49620-93-1-0148

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Bolling Air Force Base,
Washington, DC

June 30th 1994

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Time-resolved, high-resolution, x-ray microscopy of *in-vitro* biological and life science specimens with the aid of laser plasmas

As a final report for this one-year contract we submit a short summary of the success of this contract and as evidence of the technical output of this contract we submit copies of these manuscripts which have been, or are in the process of being, published. These submissions satisfy the stated requirements of the contract.

The initial intent of this contract was to help create an x-ray microscopy facility at the Laser Plasma Laboratory at CREOL specially dedicated to applications to biology. This followed on work that we had initiated in Japan. The hope of this contract was that, in setting up a dedicated facility here in the US, we might attract the collaboration of medical and biological researchers, and thereby demonstrate the usefulness of this form of x-ray analysis. Hopefully then further development of this approach could then be supported by research funds from the medical and biological sciences.

We have been successful in this endeavor. We have established a facility for biological microscopy. This facility will soon be a dedicated facility to this activity. We have produced our own x-ray images of biological specimens. Most encouragingly, we have formed collaboration with several groups of biologists and medical scientists to exploit this technology further. Principal among these are

[a] Our Japanese collaborators. These include K. Shinohara from the Tokyo Metropolitan Institute of Medical Science, Y. Kinjo of the Tokyo Metropolitan Isotope

Research Center, and K. Tanaka of the Institute of Laser Engineering of the University of Osaka. CREOL has also established a separate collaboration agreement with ILE in the general area of laser plasmas. We are currently continuing our collaboration with these workers in the area of human chromosome studies.

[b] Our British collaborators. These include Dr Tony Stead from Royal Holloway College, University of London and Dr Robin Cotton from Oxford University. We are presently studying dry and wet samples of botanical cells such as crithidia with this group.

[c] Arnold Palmer Hospital for Children & Women Orlando, Florida. We are collaborating with Dr Jayshree Rajyaaguru in the study of bacteria such as p. cerpacia that presently have a high resistance to antibiotics. This structure changes under the stimulus of certain drugs. We are using x-ray microscopy to analyze the structural effect of these drugs.

[d] Orlando Regional Health Center, Orlando Florida. We are collaborating with Dr Jane Gibson in the study of fragile X syndrome in human chromosomes.

From the above, it is evident that we have begun to make meaningful outreach to medical and biological research groups, and that we are progressing toward our goal of having the progress of this technology driven by those who stand to benefit the most from it. We hope that in the future this will lead to funding support from agencies such as NIH and NCI and outside sponsorship.

For completeness , we include copies of three publications that have so far been prepared under the tenure of this contract.

Pulsed x-ray microscopy of biological specimens with laser plasma sources.

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ABSTRACT

The development of a compact, affordable, high-resolution x-ray microscope will have a strong impact on the biological and medical sciences. We discuss the potential that pulsed, laser-plasma x-ray sources have to this development. Several approaches to the high-resolution analysis of dried and *in-vitro* biological specimens with laser-plasma sources are described. We discuss the details of the laser and plasma conditions required for optimum x-ray generation, and the various x-ray optical and x-ray electro-optical imaging systems which could be incorporated into a compact, laser-plasma x-ray microscope.

I.   Introduction.

At the present time detailed high resolution analysis of sub-cellular biological and life science structures is most commonly performed by electron microscopy. It is well-known that while this technique can provide Angstrom resolution, it suffers from a small depth of field and the need to significantly treat the samples (drying, dying, sectioning, coating etc.,) before analysis. X-ray microscopy in principle will avoid many of these limitations. While being limited to the resolution in the 100A range, it's ability to probe the internal structure of *in-vitro* assemblies provides biologists and life scientists the opportunity to observe complex features in their natural, even live state. Most x-ray microscope development has so far been made using large synchrotron sources. The latter limits x-ray microscopy to being primarily a limited research tool centered around a complex beam line located at a major synchrotron facility. The use of a laser-plasma x-ray source, however, makes plausible the development of a compact x-ray microscope having a size and cost comparable to a conventional electron microscope. The overall design of such a laser plasma-based x-ray microscope is illustrated in fig.1. One could envisage such a system being part of the standard analytical hardware available in major research and medical institutions. In this paper, we review the characteristics of laser-plasmas as they pertain to a microscope source, and discuss the various imaging technologies which can be incorporated into such a source. In the latter, we describe some new results obtained with both reflective optical microscopy and with high-resolution contact microscopy of dry and wet cellular structures. We also discuss the potential incorporation of high magnification electron-optical imaging systems into a compact x-ray microscope and the capability it will give for real-time imaging.
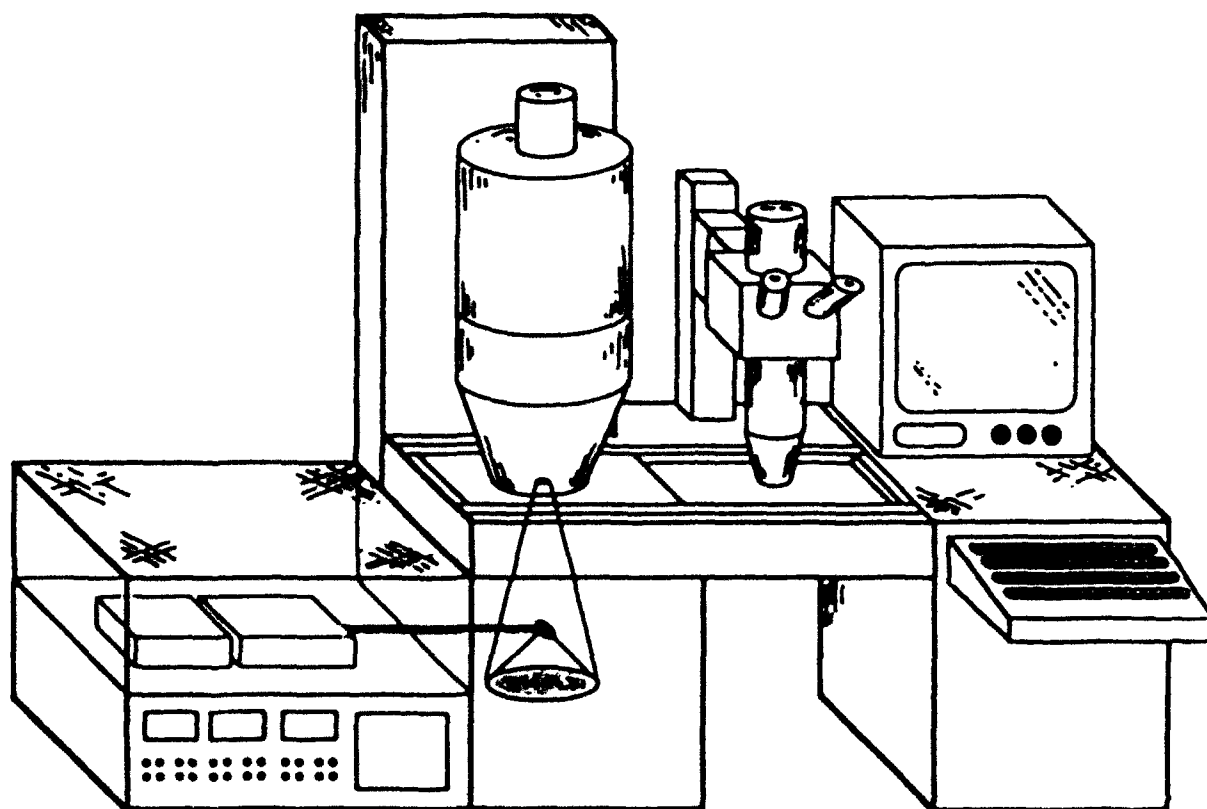
Fig.1. Design concept of a compact, stand-alone, laser-plasma x-ray microscope.

II. Advantages of a laser plasma source for x-ray microscopy of biological specimens.

The primary advantages of a laser plasma x-ray source stem from it's compactness and flexibility. As has been previously mentioned, the spectral brightness of laser plasma x-ray sources can be comparable to the brightest available synchrotrons[1]. Laser plasma x-ray sources have the advantage of being compact, moveable and tolerable of modest vacuum requirements. The x-ray emission spectrum of laser plasmas is rich in bright broad (Planckian) continuum emission and in narrow atomic emission lines, and can easily be varied to suit specific microscopy needs. Typical characteristics of the x-ray emission of laser plasmas are illustrated in fig.2. This selectivity in x-ray wavelength has a potential high dividend for x-ray microscopy in facilitating elemental analysis of features within biological structures by difference imaging with emission at two different wavelengths. Laser plasmas are point sources which can be highly reproducible, a requirement for precision x-ray optical systems having extremely high alignment specifications. Lastly pulsed laser plasmas introduce the time domain element into x-ray microscopy. Whereas exposure times for microscopy with synchrotrons are measured in seconds, laser plasmas can provide x-ray emission in pulses ranging from several nanoseconds duration to less than one picosecond. This introduces the possibility of capturing kinetic, chemical, or morphological changes in biological structures in time frames of interest to understanding complex biological processes. No other type of microscopy can offer this capability.

Many measurements of the x-ray emission from laser plasmas have been made in the spectral regions of interest for biological microscopy[2-5], that is in the so-called 'water window' (2.3-4.4nm), and at other, element specific wavelengths. However these measurements have been made with laser and

target characteristics more germane to other applications of laser plasmas (such as laser fusion and x-ray laser generation). Moreover these studies have not had reason to consider the effects of plasma and particulate blowoff from the laser plasma, an important additional issue for microscopy, as it is for the use of laser-plasmas x-ray sources for lithography[6,7], where the integrity of expensive x-ray optics in close proximity to the target must be preserved. To the author's knowledge, no detailed laser-plasma design study has yet been made specifically to satisfy all the needs of pulsed x-ray microscopy.
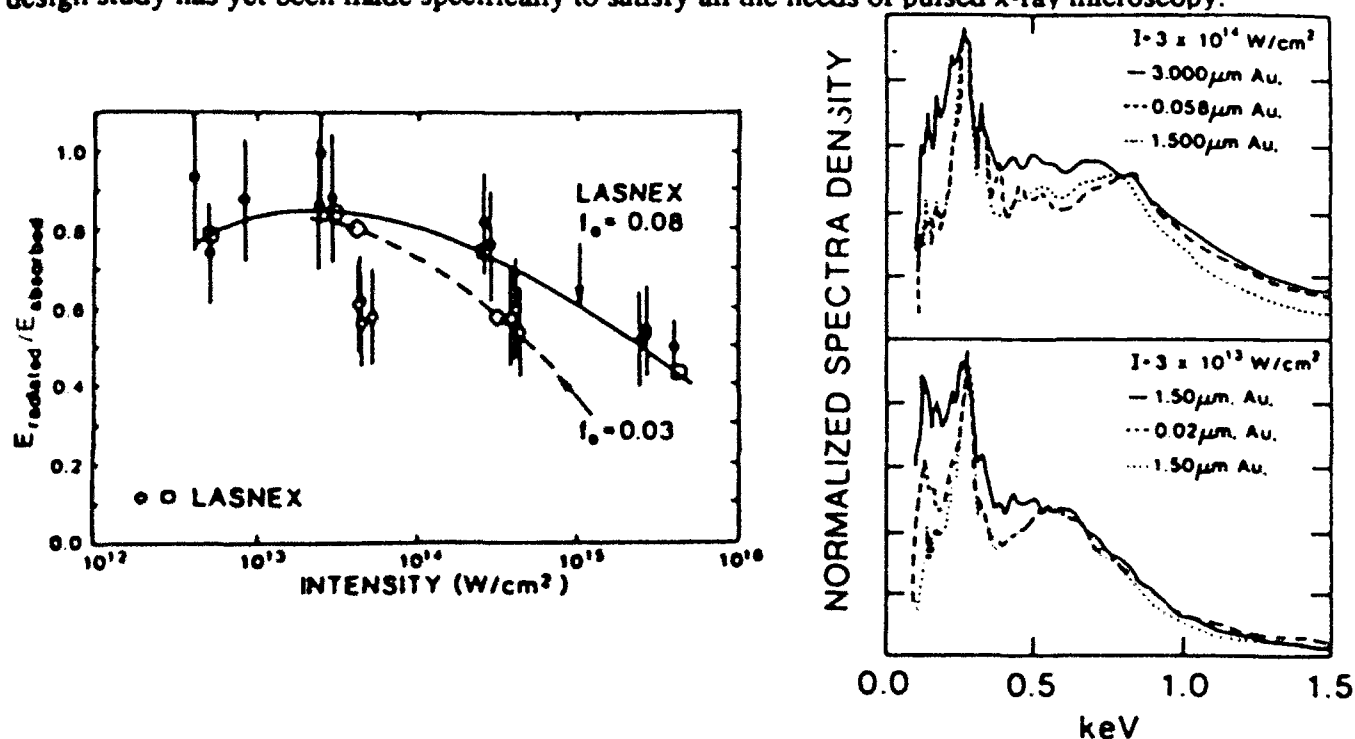

Fig.2. X-ray spectral characteristics of laser plasmas produced from Au targets. (a) shows the variation of x-ray conversion efficiency with laser intensity, and (b) indicates the variation of the x-ray spectrum with laser intensity. (from ref. 2)

III. X-ray optical requirements for a laser-plasma x-ray microscope.

In determining the specifications of x-ray optical components in a laser plasma-based x-ray microscope, we first establish the primary performance requirements of the overall system. We assume that the required resolution must be in the range of current imaging capabilities, ~50nm. This resolution, or better, has been demonstrated with Fresnel zone-plate imaging[8,9], contact imaging[10,11], and, at longer wavelengths, with normal incidence reflective optical imaging[12]. Moreover we assume that the system must be capable of real-time image acquisition. Current advanced image array detectors have pixel sizes in the 6μm range. Assuming a minimum image contrast ratio of ~10, this implies the need for an overall image magnification of ~3x10³-10⁴ This level of image magnification cannot easily be met with x-ray optics alone. Although most x-ray microscopes today rely primarily on optical elements having modest image magnification and long-time image processing of an image recorded on resist, film, or through image scanning, we believe the optimum microscope will require real-time image acquisition and thus will incorporate a combination of x-ray image magnification and electron-optical image magnification. Such a system is shown schematically in fig. 3. The specimen to be analyzed will be irradiated with monochromatic radiation from the laser plasma source. This could be facilitated by either a normal incidence multilayer mirror collector or a zone-plate condenser. An image of the specimen in the backlit radiation is then created with either a Schwarzschild microscope or Fresnel lens with a magnification (20-50) commensurate with the cathode resolution capability of an electro-optical image magnifier such as an x-ray-sensitive zoomtube[13] or an x-ray photoelectron microscope[14,15]. A zoomtube having an image

magnification of 40-200 and a cathode resolution of ~1μm has already been demonstrated[13]. Moreover, a photoelectron microscope having a resolution of 0.1μm and a magnification of 1000 should be developed in the near future[16]. At some loss in resolution, but with considerable gain in sensitivity and simplicity, the latter could be used in a simple contact or 'proximity' imaging mode.
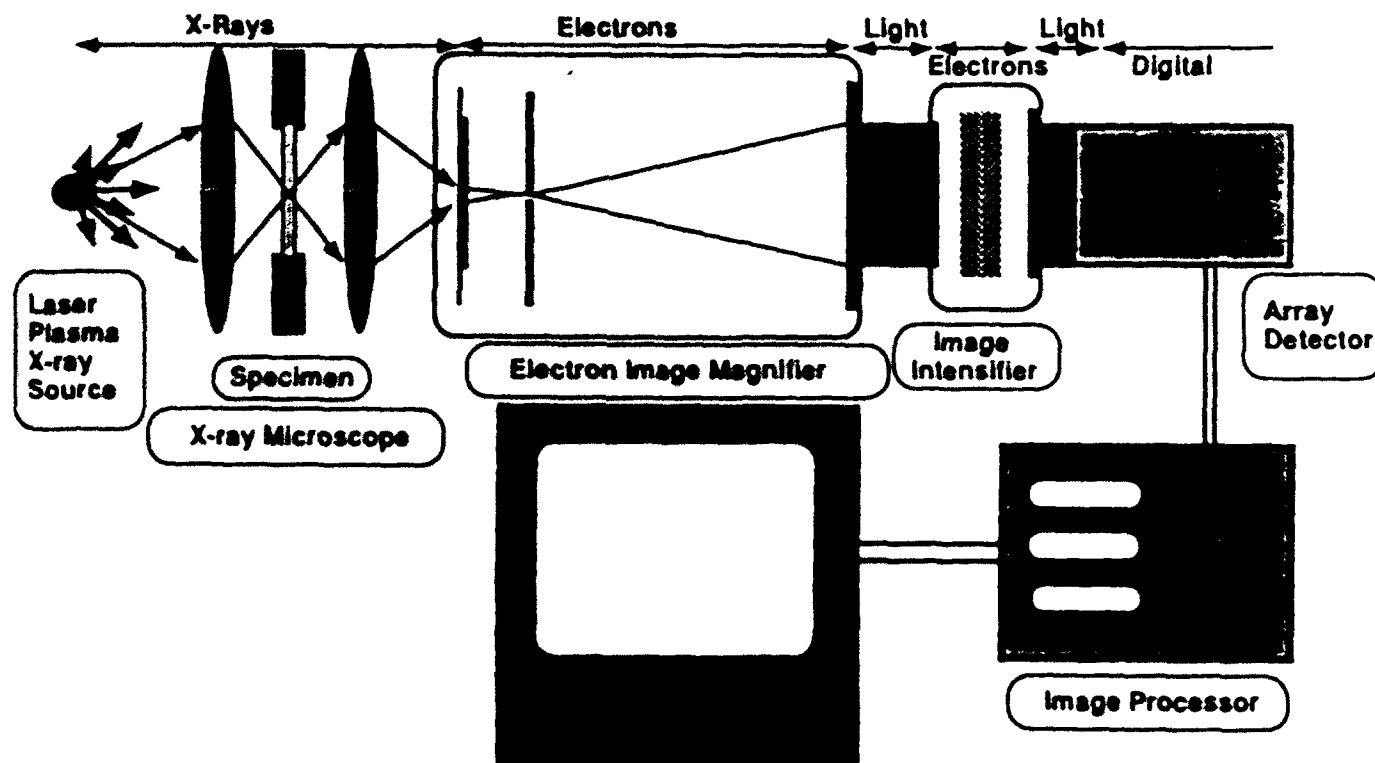

Fig.3   Possible structure of an x-ray - photoelectron-optical microscope.

We currently are investigating the practicality of two forms of x-ray imaging which could be incorporated into a laser plasma x-ray microscope of the type described above. In the first we investigate the limits of contact microscopy for analyzing in-vitro biological objects. In this investigation we study the effects of the x-radiation on the specimen, the degree to which the image contrast can be enhanced, and the resolution limits that can be achieved. In the second approach we examine the potential that Schwarzschild optics have in combination with laser-plasma x-ray sources for microscopy. We have made investigations with several Schwarzschild optics coated with x-ray multilayer coating with reflectivities centered at different wavelengths (4.42nm, 7nm and 17nm). We discuss the primary issues of resolution and contrast aswell questions of practicality, stability and ease of use in so far as envisaging such an optic in a compact x-ray microscope system.

IV.   High resolution contact imaging of in-vitro biological specimens.

Many previous studies of contact microscopy of biological and life science objects have been reported.   Several different sources have been used[17-22], including pulsed e-beam devices and synchrotrons, and a few recent investigations have been made with laser-plasma sources[21,22].

In the present studies small water specimen cells, 1-3μm thick, having a 100nm thick SiN window and backed with polymethylmethacrylate (PMMA) photoresist, fig.4(a), were used to contain stretched, live human chromosomes. They were irradiated with the x-rays generated by one pulse from a high power laser irradiating a planar Au target located 2cm away. The plasma was created on the target by 26J energy, 526nm wavelength, 1ns duration laser pulses with a focal spot intensity of ~ 10^{13}W/cm^2.

In this configuration a spherical W/C multilayer x-ray mirror[26] is used to focus 4.4nm wavelength x-rays onto the specimen cell. This approach should result in higher contrast images and in less radiation damage to the specimen from highly absorbed radiation from the x-ray source.

The principal limitation and disadvantage of this form of contact microscope is the complexity and delay involved in processing the x-ray image. This can be removed if contact microscopy could be integrated with a special form of x-ray-sensitive photoelectron microscope. Considerable progress has been made in the last few years in developing low energy photo-emission microscopes[14,15]. The spatial resolution of these instruments can now reach below 100nm[14,15]. The development of highly resolving transmission x-ray photocathodes having a spatial resolution in this range, and their incorporation with a similar electron-optical system to that used in a photo-emission microscope[16], would provide this capability. Such a system could be used in combination with contact imaging to provide high magnification, real-time images of biological specimens.

V. Normal-incidence reflective x-ray optical microscopy.

An important component in the microscope design described above is the high-resolution x-ray objective. This can, in principle, be a reflective optic, using either grazing incidence optics or normal incidence, high-reflectivity, multilayer coatings, or a transmissive Fresnel lens. We have made considerable effort in the last few years to explore the potential of x-ray multilayer-coated Schwarzschild x-ray optical elements for high-resolution biological microscopy. It is well known that this approach to high-resolution microscopy presses the limits of x-ray mirror fabrication and efficient x-ray mirror design. A 120mm diameter, NA = 0.35, 15X Schwarzschild microscope coated with Ni/C multilayer mirrors for 7nm and having a measured resolution, limited by the recording film-grain size, of 0.5μm was developed by Kado et al[27,28], and separately Richardson et al [29] developed a smaller, 33mm diameter, NA = 0.28, 15X Schwarzschild microscope coated with a W/C multilayer mirror for 4.4nm, in the socalled 'water window' (2.5-4.4nm). In the present, we report a multilayer-coated Schwarzschild microscope for 17nm, of similar design to that in ref.28. In particular, we report it's application, for the first time with a laser plasma source to the imaging of biological specimens.

The biological specimens used in these studies were dried HeLa cells in their natural state. The cells were supported on a thin plastic membrane, and irradiated with x-rays from a laser plasma created from a thin Au target. These experiments were made with the setup shown in fig.6.
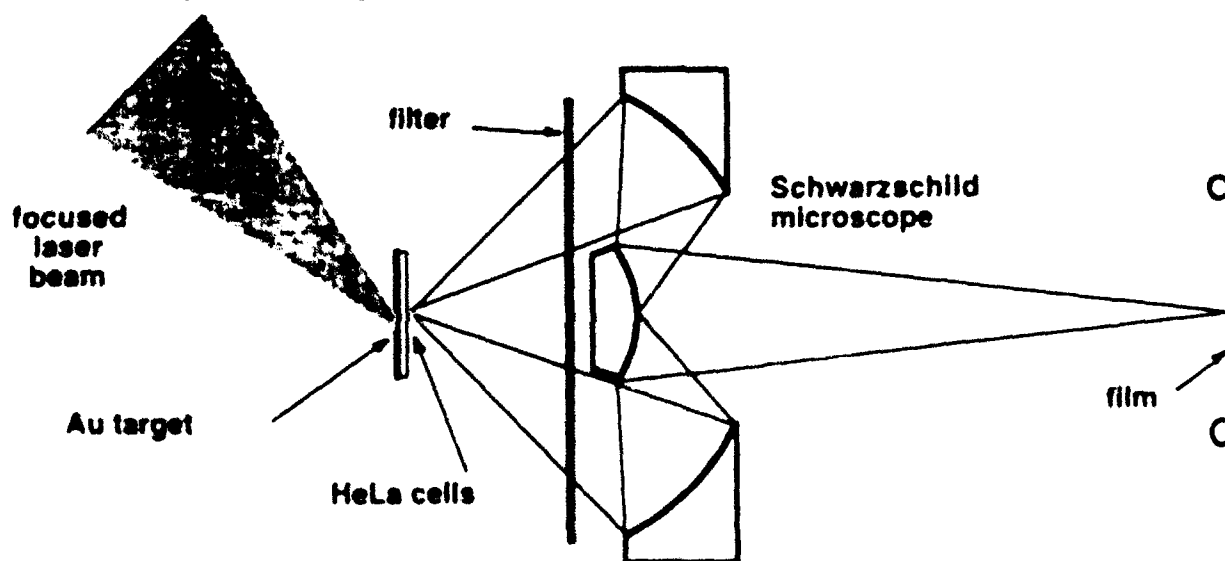

Fig.6. Experimental configuration for imaging x-ray microscopy of dried HeLa cells

he specimen cell was positioned at an angle of 45° to the normal to the target and the laser beam axis. he stretched chromosomes were prepared by the surface-spreading technique and whole-mounted irectly on the PMMA photoresist.
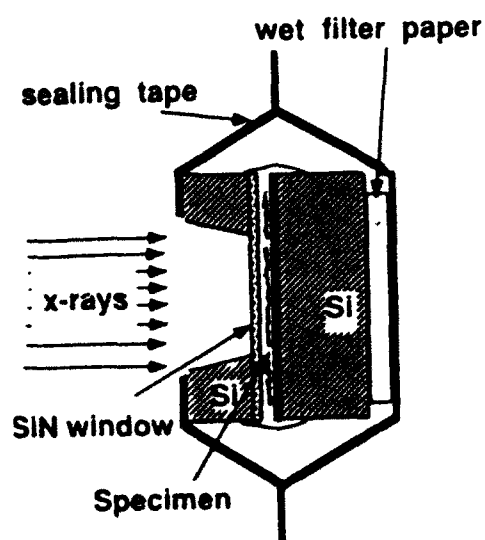

Fig.4. (a) Contact irradiation scheme. (b) Contact image of stretched in-vitro human chromosomes obtained with a single nanosecond burst of laser plasma x-rays.

After irradiation, the resist was developed by dissolving the damaged material in a mixture of methyl isobutyl ketone and isopropyl alcohol. Visualization of the three-dimensional morphology of the developed resist was then made by transmission electron microscopy of a thin ethylene replica of the resist made by plasma polymerization[23]. This technique of analysis is described in more detail elsewhere[24]. A typical image is shown in fig. 4(b). This shows details of the stretched in-vitro chromosome structure. Clearly visible are the superbead structure of chromatin fibrils, as described by Watanabe [25], composed of supertwisted DNA molecules. In fact the supertwisted DNA fiber, which has a dimension of ~10nm can be seen connecting the superbeads. This image clearly demonstrates the capability of obtaining nanosecond-time-resolved x-ray images with a resolution down to the resolution limit of the resist, using a laser plasma. Although the TEM images of these chromosomes appears quite good, we believe the image contrast can be further improved. The x-rays used to irradiate the sample in these experiments possessed a broad spectral range, not optimized for maximum image contrast of the chromosomes. This will best be achieved with x-rays in the water window. To this end we are currently using 4.4nm wavelength x-rays from a laser plasma obtained as in the configuration shown in fig.5.

Fig.5.

Scheme for monochromatic, water-window contact x-ray microscopy.
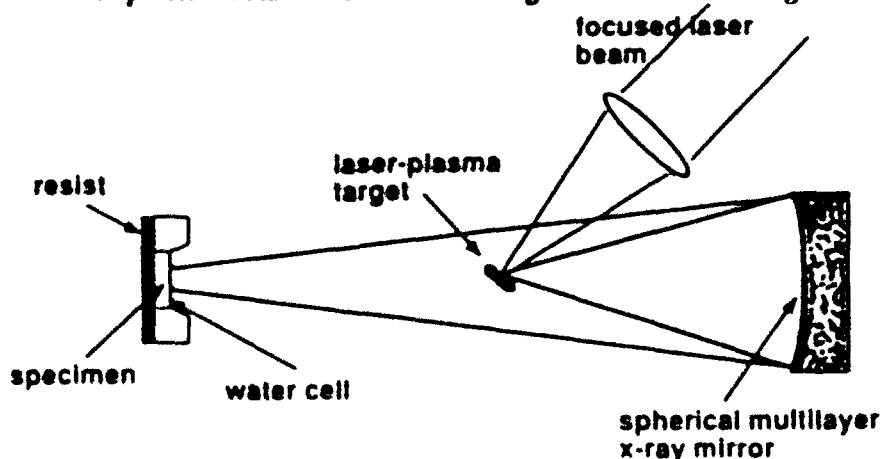
mbrane supporting the HeLa cells was configured on a rectangular grid structure in direct contact
: 0.2μm thick Au laser plasma target, the whole assembly being positioned on a metallic washer
ccurate (1μm resolution) three-dimensional stepper motor-controlled target positioner in the center
rradiation vacuum chamber at 5×10^{-4} Torr The grid structure was incorporated as an aid in
g the specimen to be in the correct position relative to the focus position of the irradiating laser,
he alignment of the Schwarzschild microscope to the specimen.
A precision method of alignment was vital to recording well-focused images of preselected
, of the HeLa cells distributed across the membrane. The position of the 200μm diameter laser
pot was accurately registered on two CCD-recorded viewing systems attached to the target
er, in separate laser irradiation experiments with thin foil targets, in which the laser plasma
ed a small ~200μm diameter hole. This hole was then used to align the lateral position of the
rzschild microscope relative to the laser plasma x-ray source. The surrogate target was then
:d with the biological cell target, and aligned optically to the laser focus position. The axial
n of the Schwarzschild microscope was then adjusted under vacuum by illuminating the grid and
:ell structure with white light and using a high-resolution CCD camera in the image plane of the
cope. precise lateral motion of the target structure them permitted selection of the desired cell
re for x-ray examination. The final x-ray image was recorded on Kodak 101 x-ray film, developed
ing to the calibration of Henke et al. A thin x-ray filter, (200nm Al on 300nm CH), was used to
visible light from the plasma from being detected. The duration of the x-ray emission is similar to
ration of the irradiating laser pulse (300ps). Thus the x-ray emission irradiate the sample before it
royed by the expanding laser plasma.

A typical single-shot x-ray image of dried HeLa cells recorded by the Schwarzschild microscope
wn in fig.7. The grid thickness and separation is 20μm and 100μm respectively. The individual,
n diameter HeLa cells are clearly visible, with a resolution of ~500nm, limited by the grain size of
film(5-7μm). Fig.8 shows the correlation between the optical image of a HeLa cell structure taken
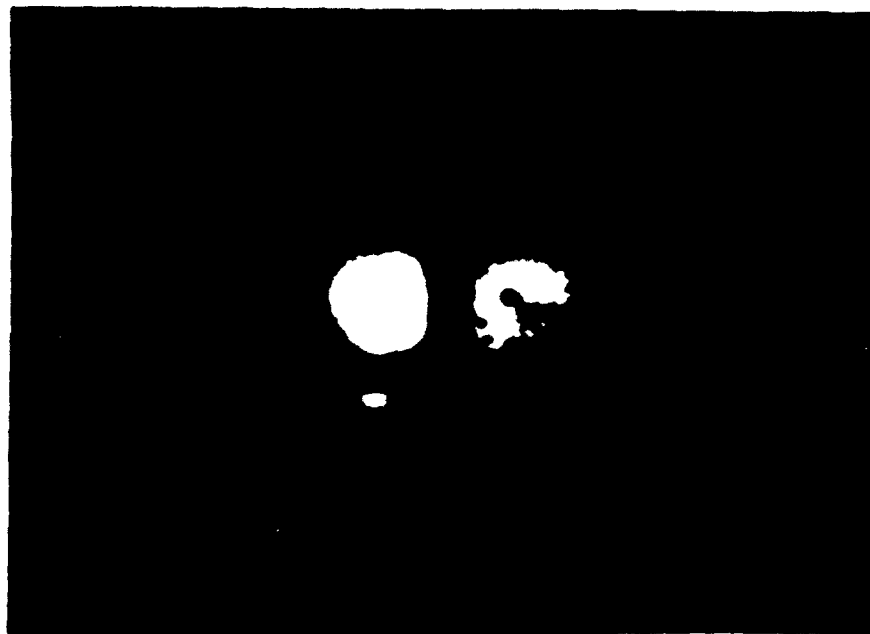o irradiation with the resulting x-ray image of the same structure.

. X-ray image at 17nm of dried HeLa cells. Grid bar width 20μm, grid separation 100μm

a   b

Fig.8.   Corresponding optical (a) and x-ray (b) images of dried HeLa cells.

These images of these biological structures are limited in resolution by the film used for image recording. This limitation will be removed in future experiments with the use of an x-ray sensitive, electron-optical zoomtube image magnifier and direct, real-time, image acquisition[13]. The full potential of this approach will then be realized.

VI.   Summary.

We have presented a concept of a practical x-ray microscope based on a compact laser plasma x-ray source.   In addition, we have summarized our progress in both contact and imaging x-ray microscopy of biological samples in their natural state.   In the future we plan to use these approaches towards two near term objectives.   Firstly these techniques will be used to survey a wider range of biological samples to demonstrate the usefulness of x-ray microscopy to biological, medical and life sciences.   Secondly, we will continue to develop these and the other technologies mentioned in this paper that are required for the successful fabrication of a practical stand-alone x-ray microscope.

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Observation of human chromosome fibers in a water layer by laser-plasma X-ray contact microscopy

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ABSTRACT

Soft X-ray contact microscopy was applied to hydrated human chromosomes. Chromosomes of human lymphocytes were spread on a clean surface of distilled water, attached on a X-ray resist, polymethylmethacrylate (PMMA), immediately covered with silicon nitride window, and mounted in a simple hydrated chamber. The specimens were exposed to a single shot of laser-produced gold plasma X-rays (600 ps) in a vacuum chamber. The developed images were observed with transmission electron microscope using the replica method with a plasma polymerization-film in a glow discharge. The results show that we have imaged the complicated entanglement of chromosome fibers in a hydrated condition. The thickness was estimated as 10 nm in average of four narrow parts of these fibers. Particle like structures were observed in many places. The present results prove that hydrated biological specimen is observable with the contrast produced by their components themselves by soft X-ray microscopy at the resolution of 10 nm.

During this imaging exposure, however, silicon nitride (SiN) window was broken. We have studied the reason for this evidence and found that the energy absorbed by the SiN window or water layer was very high. The estimated temperature increase were 870-1470 °C for SiN and 43 °C for water layer. These results suggest that the temperature increase may be responsible for the breakage of SiN window.

1. INTRODUCTION

Application of soft X-ray microscopy to hydrated biological specimens has been demonstrated in recent years [1]. Accumulation of these results will present the new findings in biology.

Chromosomes are composed of DNA, an essential genetic material in life, and proteins. In addition, chromosome fibers have a unit structure called nucleosome, a disc-like structure of 11 nm in diameter and 5.5 nm in height. Nucleosomes are made of DNA and eight core proteins called histone. They are packed to form 30 nm chromatin fibers in a interphase cell and folded into chromosome at mitosis. During a cell cycle, nucleosomes are folded to form chromosomes for cell division and unfolded to 30 nm fibers and a single nucleosome for replication and transcription. Therefore, it is of great interest to understand this highly organized process of nucleosomes to form chromosomes and chromosome fibers, especially in situ. As a step to this goal, we have imaged dry nucleosomes in chromosome fibers in a previous paper [2]. In the present report, we have tried to observe hydrated chromosome and obtained the images of unfolded chromosome fibers. The thickness of the fibers was estimated as 10 nm in narrow parts, which means that we have imaged the size of nucleosomes and suggest that X-ray microscopy will give

us the new method to understand the detailed configuration of nucleosomes in chromosome fibers.

2. MATERIALS AND METHODS

Preparation of human chromosomes are as described elsewhere [3,4]. Briefly, chromosomes from mitotic human lymphocytes (RPMI 1788) were spread on a clean surface of distilled water and whole-mounted directly on a X-ray resist, polymethylmethacrylate (PMMA). The PMMA with a water droplet was immediately mounted in a simple hydrated specimen chamber [5]. The chamber was composed of silicon nitride window (0.25 mm x 0.25 mm; thickness, 0.4 mm) and PMMA supported by silicon bases (see, Fig. 7), and covered with a sticky tape (Scotch Sealing Tape #483). The thickness of water layer was 1-5 mm. The chamber was placed at the specimen-target distance of 28.8 mm and exposed to laser-produced gold plasma X-rays (laser wavelength, 527 nm; energy, 15.9 J) with a pulse length of 600 ps at Gekko IV, Institute of Laser Engineering, Osaka University [6]. Exposed specimens were treated as described previously [3]: (1) Removing the specimen from PMMA with sodium hypochlorite (chlorine concentration, 0.5%), (2) developing the PMMA with mixed solution of methylisobutylketone and isopropanol (3:1) for 250 s, and (3) observing with transmission electron microscope using the replica method with plasma polymerization-film in a glow discharge made of a mixture gas of ethylene and methane.

3. RESULTS AND DISCUSSION

3.1. Imaging chromosome fibers

Figure 1 shows an image of stretched part of human chromosome fibers. Since replica method was used for the observation of developed PMMA, white line surrounded by dark lines corresponds to X-ray image [2] in Fig. 1. Figure 2 shows the negative image of Fig. 1. Complicated entanglement of dark lines was observed in the whole area of Fig. 2. The image with weak contrast may be the image for the entangled chromosome fibers unfolded and dispersed from a chromosome, the remainning core part of which was imaged with clear contrast. Figure 3 shows another image of a remaining chromosome core and chromosome fibers. The negative image of Fig. 3 is shown in Fig. 4. The image shown in Figs. 3 and 4 may be the image of chromosome fibers sprung out from one chromosome core.
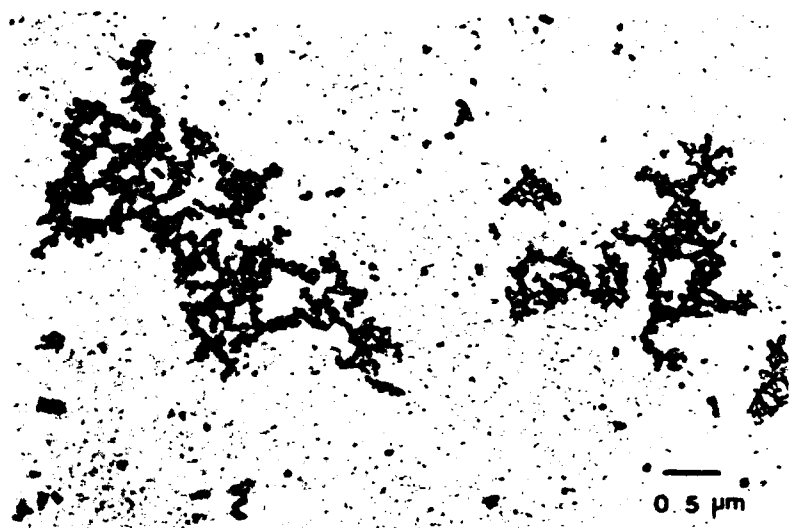

Fig. 1. X-ray image of entangled chromosome fibers with remaining core of chromosomes.
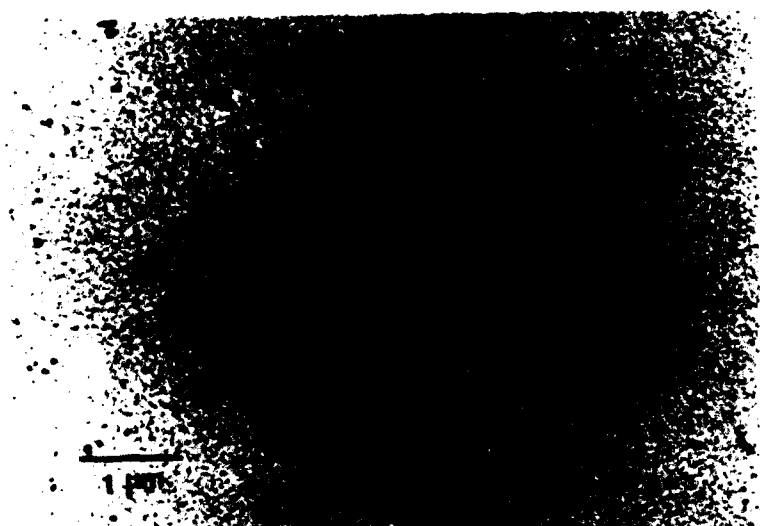
Fig. 2. Negative image of Fig. 1.

Fig. 3. Another image of entangled chromosome fibers with a remaining core of a chromosome.

1 µm

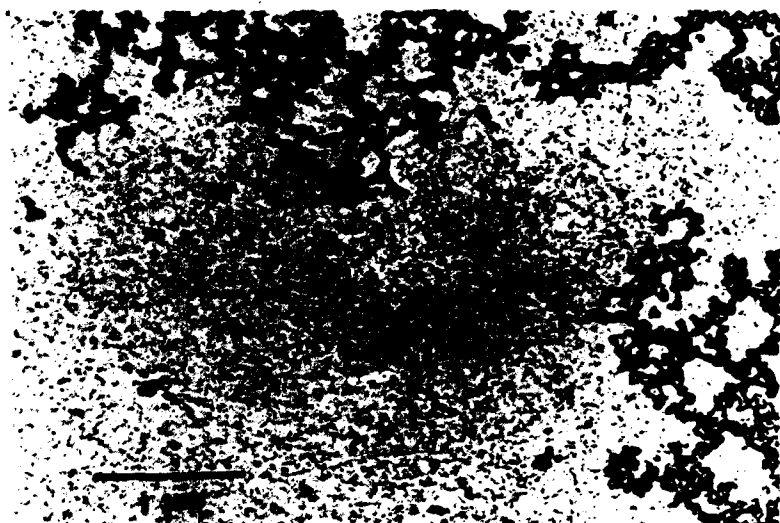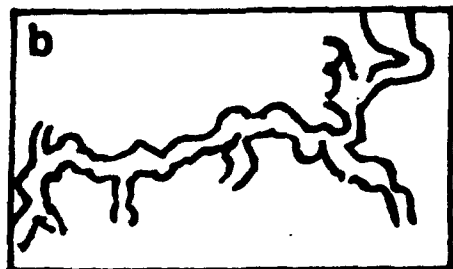Fig. 4. Negative image of Fig. 3.

Fig. 5. Different image of an
entangled chromosome
fibers.

Fig. 6a. Enlarged image of a
part of Fig. 5.
(surrounded
by a rectangle line)
6b. Illustration of a
rectangle part of
Fig. 6a.

Different part of the image of chromosome fibers was shown in Fig. 5. The rectangle part surrounded by a line was enlarged and shown in Fig. 6a. Figure 6b illustrates a part of the image in Fig. 6a. The thickness of the four narrow parts of the image of a chromosome fiber shown by arrows were estimated as 9.9 nm, 9.9 nm, 9.5 nm, and 10.7 nm (10.0±0.5 nm in average). These narrow parts show particle shapes suggesting the image of nucleosomes.

The present results indicated that chromosome fibers and their unit structure, nucleosomes in a hydrated condition were observed by soft X-ray contact microscopy using pulsed (600 ps) laser-produced plasma X-rays with the contrast of their components and

suggest that soft X-ray microscopy will be a new method to study the structure of hydrated biological specimens at the resolution of as high as 10 nm.

3.2. X-ray spectra and the dose estimates

Figure 7 illustrates the exposure system. Laser (wavelength, 527 nm) was focused to a gold target. The X-rays emitted from the gold plasma show a broad spectrum with a peak wavelength of 6.2 nm [7]. The X-rays were, however, exposed to the specimen in water after passing through SiN window. Therefore, the actual spectrum of the exposed X-rays to the specimen should be changed from that of the source because of the absorption by the SiN window and water. We have estimated the X-ray spectra of various stages from level 0 to level 2 as shown in Fig. 7 with the following equation:

$$I = I_s \cdot \exp(-\mu \cdot d) \qquad \dots\dots\dots(1)$$

where I_s and I are X-ray fluences before and after the substance (i.e., SiN window, water, or water and a nucleosome), μ and d are the linear absorption coefficient and the thickness of the substance. Since μ is dependent on the energy of a photon (wavelength of X-rays), fluence I was calculated for each photon energy by the equation (1).

Fig. 7. Illustration of the experimental system.

Figure 8 shows the spectra of the source (gold plasma; broken line), of the X-rays immediately after the 0.4 μm SiN window (level 1 in Fig. 7), and of the X-rays after passing through 1 μm water layer (level 2 without nucleosome in Fig. 7). Total photon flux (F) after the SiN window was estimated by the following equation:

$$F = \Sigma I_{Ei} \cdot \delta Ei = \Sigma[I_s \cdot \exp(-\mu_{SiN} \cdot d_{SiN})]_{Ei} \cdot \delta Ei \quad \dots(2)$$

where suffix Ei means the value at the photon energy of Ei. With the exposure condition for the Figs. 1-6 (laser energy, E_L=15.9 J; target-specimen distance=28.8 mm; thickness of SiN window d_{SiN}=0.4 μm), the total photon flux in the energy range of 0.114-1.254 keV (0.99 nm-10.9 nm) was estimated as 4.0×10^{14} photons/cm^2. Figure 9 shows the comparison of the spectrum of the X-rays after the water layer (1 μm) with that after the water (1 μm) and a long axis (11 nm in depth) of a nucleosome (level 2 of the case 2 in Fig. 7). The difference of these two spectra was shown in Fig. 10. This difference should cause the production of the image contrast of a nucleosome. The percent contribution to imaging a nucleosome of the wavelength of longer than 4.37 nm, 4.37-2.33 nm (water window), and shorter than 2.33 nm were 1.1%, 82.0%, and 16.9%, respectively. The results indicated that X-rays in water window were the main photons contributing to to the contrast formation of a nucleosome in water.

The results were in good agreement with the expectation for X-ray microscopy of hydrated biological specimens. It should be noted, however,
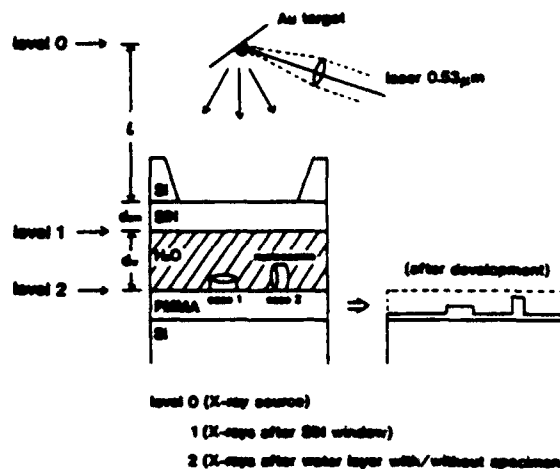

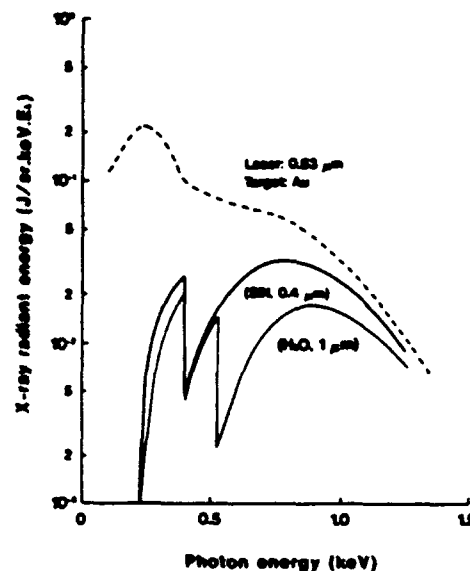
Fig. 8. X-ray spectra of a source, level 1 (after the SiN window, and level 2 (after the water layer).
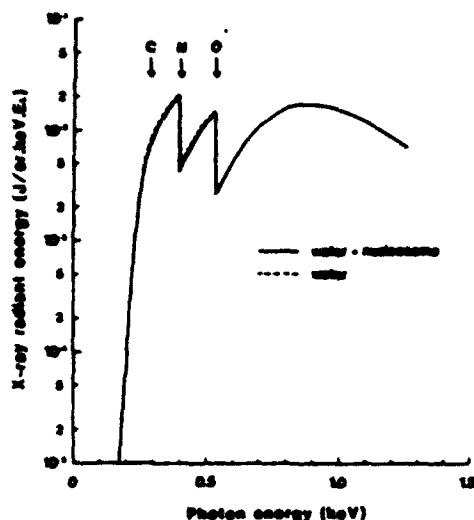
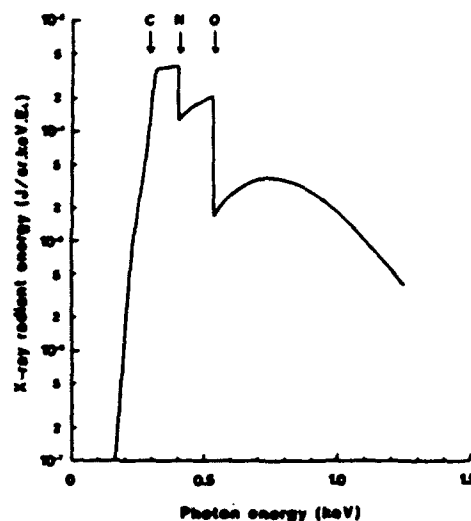Fig. 9. X-ray spectra of level 2 (after the water with/without a nucleosome).

Fig. 10. Difference in X-ray spectrum after water layer with nucleosome from the spectrum after water layer alone.

that there existed a significant amount (16.9%) of contribution by X-rays shorter than 2.33 nm to the image contrast.

We have noticed that the SiN window was broken by a single shot of exposure. To study the cause for this evidence, we have estimated the energies absorbed by the SiN window and by the water with the following equations:

$$E_{SiN} = \Sigma[I_s \cdot \{1-\exp(-\mu_{SiN} \cdot d_{SiN})\}]_{E_i} \cdot \delta E_i \qquad \ldots\ldots\ldots\ldots(3)$$

for SiN window and

$$E_{H2O} = \Sigma[I' \cdot \{1-\exp(-\mu_{H2O} \cdot d_{H2O})\}]_{E_i} \cdot \delta E_i \qquad \ldots\ldots\ldots\ldots(4)$$

for water layer where I' is X-ray fluence after SiN window. In the present exposure condition for Figs. 1-6 (laser energy, E_L=15.9 J; target-specimen distance=28.8 mm; thickness of SiN window, d_{SiN}=0.4 μm), estimated energies were as follows:

E_{SiN} = 0.134 J/cm² at 0.4μm SiN, and
E_{H2O} = 0.018 J/cm² at 1 μm H_2O.

The results may correspond to the temperature increase (δT) for SiN window of,

δT_{SiN} = 1470 °C if we use C_p = 99.89 J/K·mol at 25 °C or
　　 = 869 °C if we use C_p = 169 J/K·mol at 727 °C, and for water layer of,
δT_{H2O} = 43 °C

where C_p is a molar heat at constant pressure assuming that there is no thermal loss during the exposure time of 600 ps. Therefore, the cause for the breakage of SiN window may be attributed to the temperature increase in SiN window and water in addition to the physical damage caused by plasma particles. The use of monochromatic X-rays in water window may reduce these temperature increase remarkably and prevent the breakage of SiN window.

4.CONCLUSION

Human chromosome fibers were observed in hydrated condition with a sigle shot of flush X-ray contact microscopy with a laser-produced gold plasma X-rays. The image showed the complicated entanglement of chromosome fibers unfolded and dispersed from a chromosome. The thickness of the narrow parts of unfolded chromosome fibers was estimated as 10 nm

corresponding to the size of a nucleosome. The present results prove that hydrated biological specimen is observable with the contrast produced by their components themselves by X-rays. The studies on the X-ray spectra of the imaging and the dose estimates indicated that there may be a big temperature increase in SiN window and recommended the use of monochromatic X-rays at the wavelength in water window for the imaging of nucleosomes, a unit structure of a chromosome.

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Development of X-Ray microscopy systems based on laser plasma sources

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ABSTRACT

We report progress in two areas of technology relevant to the development of high-resolution x-ray microscopy techniques based on laser plasma x-ray sources for the analysis of wet and dry biological specimens. The approach we discuss involves the use of ultrashort x-ray emission from a laser plasma source. Precision x-ray optics are used to collimate and filter this light onto the specimen. Imaging and image magnification are accomplished with a combination of x-ray and electron-optical systems. We discuss our progress towards establishing a flexible laser-plasma x-ray source for the development of biological imaging and contact microscopy, and progress we have made towards the development of an electro-optical imaging system having high magnification and spatial resolution.

1. Introduction.

X-ray microscopy in principle will avoid many of the limitations of present-day electron microscopy techniques for analyzing biological material. Principal among these are the small depth of field and the need to significantly treat samples (drying, dying, sectioning, coating etc.,) before analysis. While being limited to the resolution in the 100A range, the ability of x-ray microscopy to probe the internal structure of *in-vitro* assemblies provides biologists and life scientists the opportunity to observe complex features in their natural, even live state. Most x-ray microscope development has so far been made using large synchrotron sources. The latter limits x-ray microscopy to being primarily a limited research tool centered around a complex beam line located at a major synchrotron facility. The use of a laser-plasma x-ray source, however, makes plausible the development of a compact x-ray microscope having a size and cost comparable to a conventional electron microscope. We have previously discussed several approaches towards x-ray microscopy based on laser plasma x-ray sources[1]. In this paper we describe progress we have made at the Laser Plasma Laboratory at CREOL towards constructing a flexible laser-plasma-based facility for the development of x-ray microscopy techniques, and the progress we have made in electron-optical image tube technology that will hasten the development a compact real-time imaging system for x-ray microscopy.

## 2. A laser-plasma x-ray source for the development of x-ray microscopy.

The primary advantages of a laser plasma x-ray source for x-ray microscopy system from it's compactness and flexibility. The spectral brightness of laser plasma x-ray sources can be comparable to the brightest available synchrotrons[2]. Laser plasma x-ray sources have the advantage of being compact, moveable and tolerable of modest vacuum requirements. Compared to synchrotrons they are much cheaper to install and to operate. The x-ray emission spectrum of laser plasmas is rich in bright broad continuum emission and in narrow atomic emission lines,

and can easily be varied to suit specific microscopy needs. This selectivity in x-ray wavelength has a potential high dividend for x-ray microscopy in facilitating elemental analysis of features within biological structures by difference imaging with emission at two different wavelengths. Laser plasmas are point sources which can be highly reproducible, a requirement for precision x-ray optical systems having extremely high alignment specifications. Lastly pulsed laser plasmas introduce the time domain element into x-ray microscopy. Whereas exposure times for microscopy with synchrotrons are measured in seconds, laser plasmas can provide x-ray emission in pulses ranging from several nanoseconds duration to less than one picosecond. This introduces the possibility of capturing kinetic, chemical, or morphological changes in biological structures in time frames of interest to understanding complex biological processes. Moreover the influence of radiation damage on image reliability is avoided by using exposure times short enough to prevent the sample responding to the damaging radiations. No other source for x-ray microscopy can offer this capability.

Although many measurements of the x-ray emission from laser plasmas have been made in the spectral regions of interest for biological microscopy[3-6], that is in the so-called 'water window' (2.3-4.4nm), and at other, element specific wavelengths., many of them have been made with laser and target characteristics more germane to other applications of laser plasmas. Moreover these studies have not had reason to consider the effects of plasma and particulate blowoff from the laser plasma, an important additional issue for microscopy, as it is for the use of laser-plasmas x-ray sources for lithography[7,8] where the integrity of expensive x-ray optics in close proximity to the target must be preserved. At CREOL we have initiated a broad program of development of laser plasma x-ray sources for lithography and microscopy. This program includes addressing not only the efficient generation of x-ray emission at specific wavelengths, but also addressing the issues of target debris mitigation and inhibition, and the engineering issues associated with routine high repetition rate operation that are required for many of these applications. For x-ray microscopy of biological material, we have commenced a detailed study of optimum laser plasma conditions for constructing a source of x-rays in the water window and at shorter wavelengths. The laser system that we will use for these studies is shown in Fig.1.

Fig.1. 700 MW solid state laser incorporating nonlinear wavefront correction by stimulated Brillouin scattering (SBS.
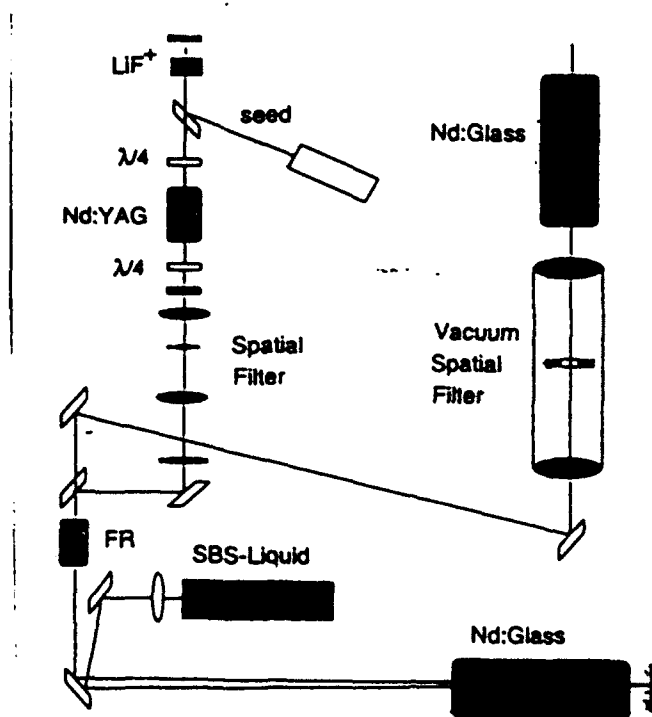
The laser incorporates an oscillator followed by several amplifier units in a fully imaged optical beamline. The Nd:YAG oscillator is Q-switched with a solid-state passive saturable absorber (LiF+) [9], and is frequency injection locked with the output of a diode-pumped cw Nd:YAG laser. Its output consists of a 12 ns duration laser pulse in a diffraction limited beam of peak power 1 MW. The output of this laser is imaged-relayed through an air spatial filter to a four-pass amplifier which utilizes a Faraday rotator (FR) as the final output element. This amplifier stage also incorporates a phase-conjugation mirror which preserves the wavefront quality of the output of the oscillator as it passes through four consecutive passes of the 410 mm long Nd:glass amplifier. The 150 MW output of this amplifier is them relayed through a 1:1 vacuum spatial filter to a single-pass 16 mm diameter 410 mm long amplifier. The output of the laser has a peak power of up to 700 MW with a pulse duration of ~7 ns, the [pulse-shortening being due to the influence of of gain saturation and the nonlinearity of the phase conjugation mirror[10]

Experiments will be carried out in the experimental chamber shown in Fig.2. The laser beam is focused onto targets with a f = 14 cm focal length lens, producing intensities in the range $1 \times 10^{12} - 5 \times 10^{13}$ W/cm^2. Biological samples are exposed to x-rays from the target by encapsulating them in a hydrated cell of the type shown in Fig.2(b), situated 2 cm from the target. an array of x-ray diagnostic instrumentation including x-ray diodes, an x-ray crystal spectrograph, an x-ray pinhole camera and a flat field grating spectrograph is used to characterize the x-ray radiation.
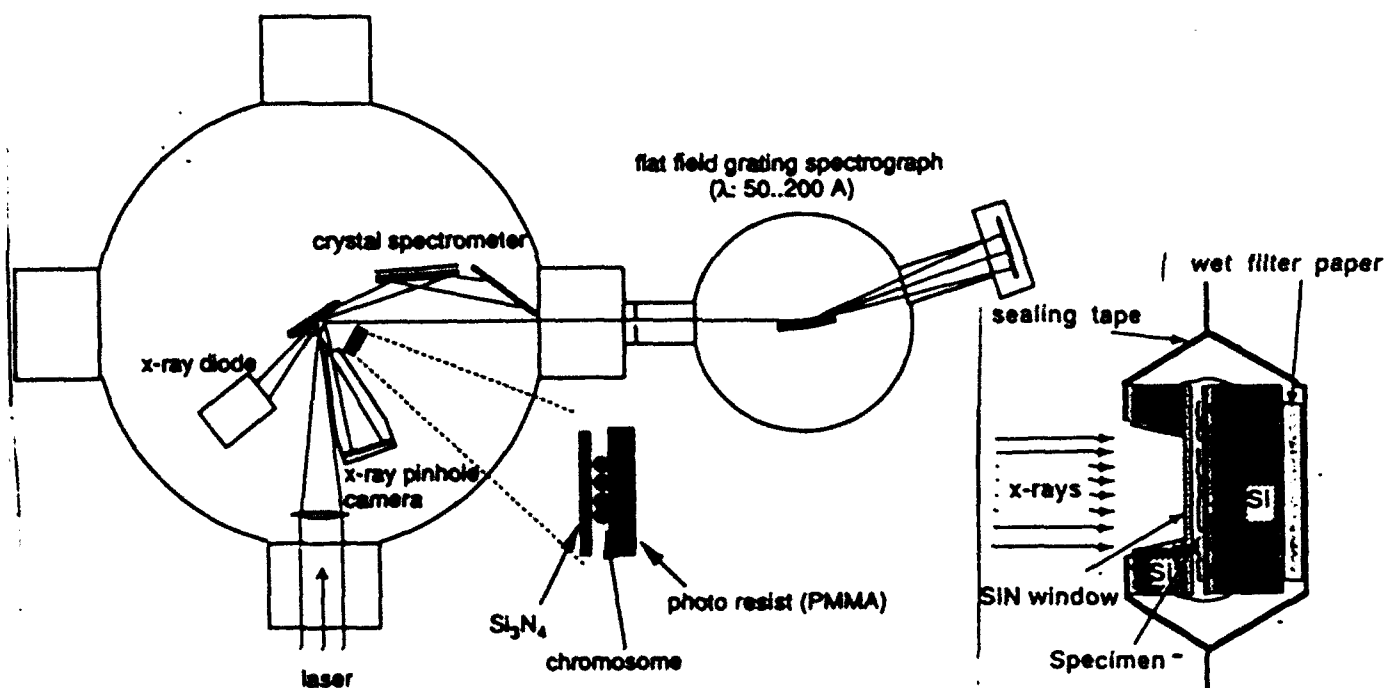

Fig.2. (a) Experimental set up used for initial biological x-ray microscopy experiments at CREOL. (b)Hydrated cell for exposing biological material for contact imaging.

3. A high resolution, high magnification electron-optical x-ray imaging system.

In determining the specifications of x-ray optical components in a laser plasma-based x-ray microscope, we first establish the primary performance requirements of the overall system. We assume that the required resolution must be in the range of current imaging capabilities, ~50nm. This resolution, or better, has been demonstrated with Fresnel zone-plate imaging[11,12], contact imaging[13,14], and, at longer wavelengths, with normal incidence reflective optical imaging[15]. Moreover we assume that the system must be capable of real-time image acquisition.

Current advanced image array detectors have pixel sizes in the 6μm range. Assuming a minimum image contrast ratio of ~10, this implies the need for an overall image magnification of 10^3-10^4. This level of image magnification cannot easily be met with x-ray optics alone. Although most x-ray microscopes today rely primarily on optical elements having modest image magnification and long-time image processing of an image recorded on resist, film, or through image scanning, the optimum microscope will require real-time image acquisition and thus will incorporate a combination of x-ray image magnification and electron-optical image magnification. Such a system is shown schematically in fig. 3. The specimen to be analyzed will be irradiated with monochromatic radiation from the laser plasma source. This could be facilitated by either a normal incidence multilayer mirror collector or a zone-plate condenser. An image of the specimen in the backlit radiation is then created with either a Schwarzschild microscope or Fresnel lens with a magnification (20-50) commensurate with the cathode resolution capability of an electron-optical image magnifier such as an x-ray-sensitive zoomtube[16] or an x-ray photoelectron microscope[17,18]. A zoomtube having an image magnification of 40-200 and a cathode resolution of ~1μm has already been demonstrated[16]. Moreover, a photoelectron microscope having a resolution of 0.1μm and a magnification of 1200 should be developed in the near future[19]. At some loss in resolution, but with considerable gain in sensitivity and simplicity, the latter could be used in a simple contact or 'proximity' imaging mode.
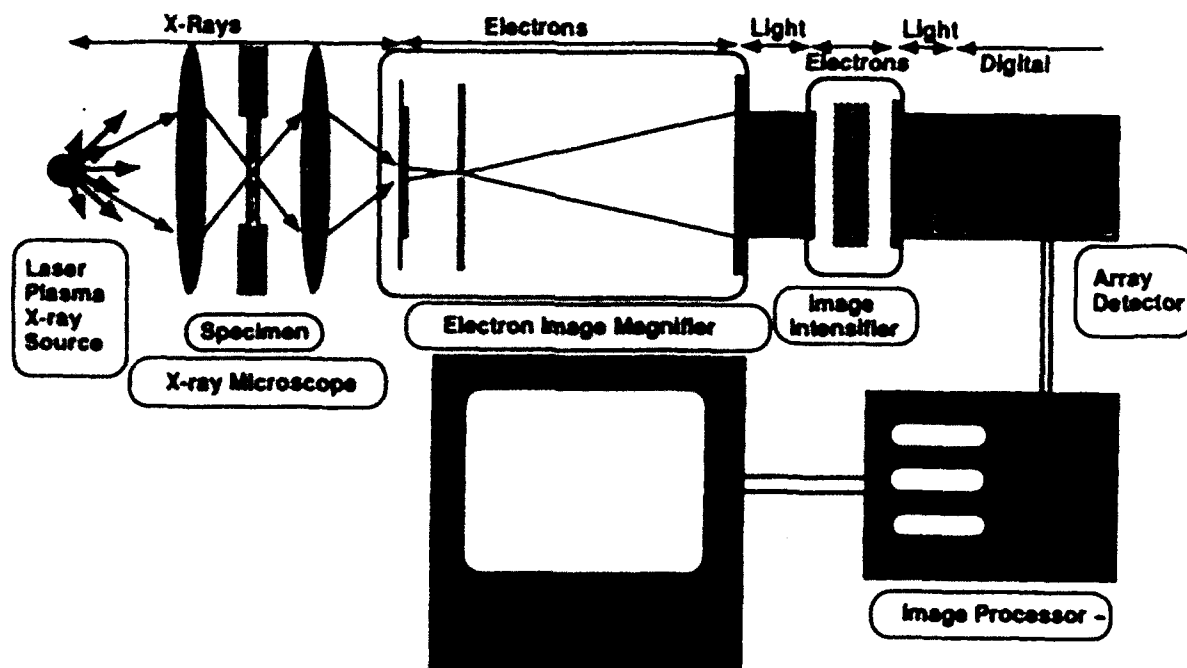

Fig.3   Possible structure of an x-ray - photoelectron-optical microscope.

An electron-optical image magnification tube of the type shown in Fig.3. is currently being built at the National Institute for Standards and Technology (NIST) for use with the SURF II synchrotron light source in a microscope for the observation of masks made for the optical alignments of soft x-ray lithography systems[20]. We are participating in this development by developing the high resolution soft x-ray photocathodes required for this system. These photocathodes must resolve features of 0.1 μm at a wavelength of 13.5 nm. We have therefore assembled at the Laser Plasma Laboratory at CREOL a dedicated x-ray photocathode fabrication

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facility for the development of efficient x-ray photocathodes for high resolution electron-optical systems. We have also tested some of these new types of photocathodes on a calibrated beamline of SURF II[21]. Fig.4 shows the photo-emissive yield in electrons/photon in the wavelength range adopted for the NIST Conversion Microscope for a number of photocathode materials.
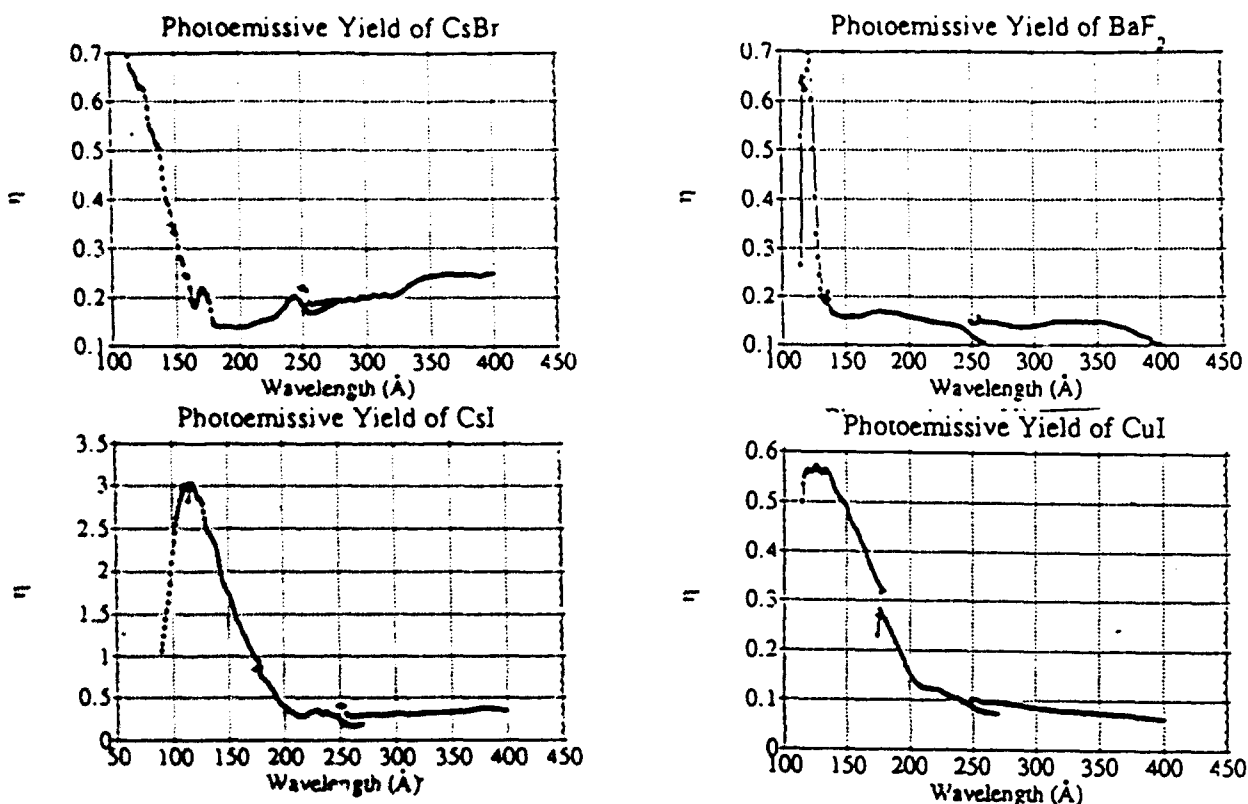

Fig. 4 Photoemissive yields of various photocathodes as measured on SURF II.

We intend to adapt the design of this electron-optical imaging system for applications to biological imaging. Although the electron optical imaging system will remain the same, we will develop new x-ray photocathodes for the water window and shorter wavelengths.

4. Summary

We have presented in this paper the progress we have made toward the development of x-ray microscope technology based on laser plasma point x-ray sources. We have established at the Laser Plasma Laboratory at CREOL a solid state laser system dedicated to the development of optimum point sources for biological x-ray microscopy. This source is currently being used to make progress in the use of contact microscopic techniques, and will also soon be used for advanced x-ray imaging systems incorporating multilayer coated Schwarzschild optics and high resolution zone plates. Towards the development of high resolution, high magnification electron-optical imaging systems for x-ray imaging applications we have established a dedicated x-ray photocathode fabrication facility and wish soon to adapt electron-optical designs of the soft x-ray conversion microscope at NIST for a similar instrument sensitive for x-rays in the water window and of shorter wavelength. These developments will bring closer the realization of a compact real-time x-ray imaging microscope for the biological and life sciences.

5. Acknowledgments

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Direct Imaging in a Water Layer of Human Chromosome Fibres Composed of Nucleosomes and Their Higher-Order Structures by Laser Plasma X-ray Contact Microscopy

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RUNNING TITLE: Imaging Hydrated Human Chromosome Fibres

ABSTRACT

X-ray contact microscopy with a 300 ps duration laser-plasma X-ray source has been used to image hydrated human chromosome fibres. Clearly imaged are individual nucleosomes and their higher order particles (superbeads), elementary chromatin fibrils of ~30 nm in diameter and their higher order fibres of various sizes up to ~120 nm in diameter. The results demonstrate that X-ray microscopy is now capable of opening a new path of investigation into the detailed structures of hydrated chromosome fibres in their natural state.

INTRODUCTION

Soft X-ray microscopy has many potential advantages over other microscopies for the visualization of thick, hydrated biological specimens in the resolution range 10-100 nm such as ① better resolution than optical microscopy and ② higher penetration depth than electron microscopy (Kirz and Sayre, 1980). These advantages have lead to an increasing effort in X-ray microscope developement, particularly in the last few years. Several approaches to high resolution imaging are now being tried, and have already demonstrated the potential of this new microscopy in visualizing different biological structures. These include the observation of proteoglycan (Panessa *et al.*,1981), myosin filaments (Panessa-Warren, 1984), living platelets (Feder *et al.*,1985) sea urchin sperms (Tomie *et al.*,1991) and human chromosomes (Shinohara *et al.*,1992a) by contact microscopy, midge polytene chromosomes by imaging zone plate microscopy (Guttmann *et al.*,1992), and bean chromosomes by scanning zone plate microscopy (Williams *et al.*, 1992). The latter two methods have not demonstrated sufficient resolution yet to reveal the fine structures of chromatin fibres including nucleosomes.

Observing fine structures of chromosome and chromatin as intact as possible in a hydrated state is important for better understanding their organization and functions *in situ.* These functions may play a key role in various cellular

control mechanisms such as cell cycle progress, differentiation, radiation sensitivities and cell death.

Eukaryotic chromosomes are composed of chromatin fiberes which themselves consist of unit structures (Watanabe *et al.*, 1990), nucleosomes of 10-15 nm in diameter (Langmore and Wooley, 1975; Oudet *et al.*, 1975) and superbeads of 15-50 nm in diameter (Hozier *et al.*, 1977; Azorin *et al.*, 1982; Zentgraf and Franke, 1984). Bordas *et al.* (Bordas *et al.*, 1986) have shown that the native uncondensed chromatin fibre in solution has an outer diameter of ~30 nm using synchrotron radiation scattering analysis. Belmont *et al.* (Belmont *et al.*, 1987) have demonstrated that size hierarchy of discrete chromatin structural domains with cross-sectioned diameters of 120, 240, 400-500, and 800-1000 Å exist in mitotic chromosomes by the analyses of three dimensionally reconstructed electron microscopic images of isolated *Drosophila melanogaster* chromosomes. Neverthless, the organization and functions of chromosomes or chromsatin*s* in *situ* have not been clarified yet.

In a previous publication (Shinohara *et al.*, 1990), we have described the first use of X-ray contact microscopy to observe the "beads-on-a-string" structures in human chromosome fibres. The chromosome fibres were as close to their natural state as possible. Namely, they were unstained without any fixative. However they were dried, and that the drying process in some way may have modified the fine structure of chromosome fibre. This possible limitation is overcome in the study of hydrated specimens.

For the observation of hydrated biological specimens at a high resolution the exposure time should be less than 1 ms to eliminate the image brurring caused by thermal diffusion and/or radiation damage (Shinohara and Ito, 1991; Ito and Shinohara, 1992). From this point of view a laser plasma X-ray source (Rosser *et al.*, 1985) is the most suitable for X-ray microscopy at the present time.

Laser-plasma X-ray sources have previously been used for a number of contact microscopy studies of biological specimens (Tomie *et al.*, 1991; Ford *et al.*,

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1992; Cheng *et al.*, 1992) including our previous studies (Shinohara *et al.*, 1992a; Shinohara *et al.*, 1992b; Shinohara *et al.*, in press), in which natural hydrated human chromosome fibres were imaged with soft X-ray contact microscopy illuminated by a pulsed (300 ps), single burst of X-rays from a laser-plasma x-ray source. In the present work, we have precisely analysed the results obtained by the X-ray contact microscopy of human chromosome fibres in a water layer with laser produced plasma X-rays.

MATERIALS AND METHODS

Chromosome preparation in a hydrated specimen chamber

The chromosome specimens were prepared for X-ray imaging in the following way. Human lymphocytes (RPMI 1788) were incubated at 37 ℃ in RPMI 1640 medium supplemented with 10 % fetal bovine serum in the presence of 0.05 μg/ml colcemid for 16 hrs to accumulate mitotic cells. After centrifugation the cells pellet was placed on a clean surface of distilled water. At this moment the cells were broken, with the consequence that the chromosomes rapidly flowed out across a large surface area of the water. The chromosomes were then concentrated in density on the surface of the water by decreasing the surface area over which they could float. Finally, they were whole-mounted directly onto a thin layer of photoresist, polymethylmethacrylate (PMMA), supported on a 0.4 mm thick silicon wafer. The thickness of PMMA was 0.7 μm and the overall substrate size was about 7 mm ×7 mm.

The hydrated specimens without fixation and staining were then configured into a simple hydrated specimen chamber (Shinohara *et al.*, 1992b). This is shown schematically in Fig.1. This specimen chamber was assembled in the following way. After the hydrated chromosomes were mounted on the PMMA resist [Fig.1(a)] , they were immediately covered with a thin (400 nm thick) SiN square (250 μm ×250 μm) window, ensuring that they were always maintained in a hydrated condition [Fig.1(b)] . The small SiN window had been chemically etched into a 0.4 mm thick Si wafer having approximate dimensions

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of 7 mm × 7 mm. While still in a wet state, these two wafers were then press-fitted together using a torque gauge (1.2 kgf·cm) attached to a micrometer [Fig. 1 (c)]. Thus the chromosomes were encapsulated in a fully hydrated state. The exess water was removed with a piece of a filter paper, and the system was sealed with two pieces of adhesive tape (Scotch tape #483) as shown in Fig.1 (d). A small piece of wet filter paper was included on the rear of the specimen holder in order to verify that the specimen was still hydrated at the time of X-ray exposure. The final thickness of each water cell was in the 1-5 μm range, measured with an optical microscope. Specimen chambers constructed in this manner were found to be water-tight for many hours in a vacuum chamber.

X-ray exposure

The encapsurated human chromosomes were exposed to a single burst of X-rays from a laser-produced plasma. The plasma was created from solid Au targets by the focused second harmonic radiation (527 nm) converted from one beam of the four beam GEKKO IV Nd:glass laser system at the Institute of Laser Engineering at Osaka University. The 300 ps (FWHM) duration Gaussian output pulse of energy 26 J was focud in an evacuated target chamber (10^{-4} Torr) with a spot size of 100 μm in diameter on the Au target, providing an irradiating intensity of $\sim 10^{15}$ W/cm^2. The X-ray emission from Au targets under these irradiation conditions has been well-characterized (Kodama et al., 1986). The specimen cell was situated 2 cm from the target at an angle of 25° to the laser axis and the target normal. Fig.2 shows the spectrum of X-ray flux from the plasma emitted in this direction and the spectrum of the X-rays irradiating the chromosomes taking account of the absorption of the SiN window (Henke et al., 1982). The total photon flux irradiating the specimen was estimated to be $\sim 1.4 \cdot 10^{15}$ photons/cm^2 in the photon energy range of 250 -1250 eV.

Development and Observation

After the exposure to the laser-plasma X-rays, the specimen chamber was

removed from the target chamber and was disassembled. The PMMA was then developed and teated in a procedure previously described (Shinohara *et al.*, 1986). Chromosomes were removed from the PMMA with sodium hypochlorite (chlorine concentration, 0.5%). Then the PMMA was developed with a mixture of metylisobutylketone and isopropanol. The three-dimensional topological representation in the PMMA of the absorption of the hydrated chromosome structure was then observed through the use of so-called "replica method" (Tanaka, 1983; Karasaki and Tanaka, 1984) and a transmission electron microscope(TEM). In the replica method the fine topological shape etched into the PMMA resist is transferred to a uniform-thickness film made of ethylene by plasma polymerization in a glow discharge. This structure can then be observed with a TEM.

RESULTS

Figs.3 and 4 show X-ray images of condensed chromosome fibres, and Figs.5 and 6 are images of partially decondensed chromosome fibres. The specimens were always in a hydrated condition during the exposure. In the replica method, the X-ray image from the TEM corresponds to the white lines covered with the black area(Shinohara *et al.*, 1990). Pictures marked with letters b to f show enlarged images of the framed areas of the picture a in each figure. Usually the fibres were entangled three dimensionally in a complicated manner. However, wide variety of hierarchical organization of fibres and particles are observed: nucleosomes (arrowheads), superbeads (supranucleosomal particles; black arrows), superbeads clusters (white arrows), polynucleosome filaments (thin white arrows), " 30 nm" fibers (thick white arrows), and higher order fibres with the sizes of up to 100 nm or more in diameter (Large and thick white arrows). In most images of the higher order structures, nucleosomes are identified as components. In Fig. 3(b) and Figs. 6(c,e), superbeads and the clusters of them are apparently composed of multiple nucleosomes in a " moluroid organization (Zentgraf and Franke, 1984) " The mean diameters of

nucleosomes and superbeads at various portions were estimated to be 12.4± 1.8 (n=25) and 33.6 ± 8.3 (n=35) nm, respectively. The values correspond well with those estimated for dried specimens (Shinohara *et al.*, 1990). Series of nucleosomes are considered to be polynucleosome filaments [Figs.3(c,d), 4(b) and 5(d)] , though internucleosomal linker DNA (2 nm in diameter) is not identified because of resolution limit of the technique. 30nm fiberes are seen in Figs. 3(d), 4(b), 5(c,e), and 6(b,d) where nucleosome- and/or superbead -sized particles are apparent. In one case [Fig.5(e)] , the fibre is constructed with regularly arranged two pararell rows of nucleosomes (thick white arrow). Higher order fibres constructed with 30 nm fibres are seen in Figs.5(b) and 6(b,e). In Figs. 6(b,d), fibres with diameters of ≧30 nm coil helically into thicker (60-120 nm in diameter) fibres (framed by the rectangles and the illustrations of those fibres are shown in the column of each picture). Another example of a helical coil is observed in Fig.5(b). In this case, however, the fibre coils so tightly that it is hard to estimate the internal structures.

DISCUSSION

In the present study we have succeeded in observing fine structures of human chromosome fibres in a hydrated condition by X-ray contact microscopy with the aid of laser produced plasma X-rays. The data revealed wide variety of hierarchical organization of chromosome fibres, which are fundamentally compatible with previous data (Langmore and Wooley, 1975; Oudet *et al.*, 1975; Hozier *et al.*, 1977; Azorin *et al.*, 1982; Zentgraf and Franke, 1984; Bordas *et al.*, 1986; Belmont *et al.*, 1987; Shinohara *et al.*,1990). One of those examples is "moruloid" organization of superbeads with nucleosomes which was reported by Zentgraf and Franke(Zentgraf and Franke, 1984) using conventioal electron microscopy with fixation and staining. The present study presented the first example to demonstrate such structure of unfixed, unstained and hydrated chromosome fibres. Since the chromosome fibres were spread over the surface of

distilled water, there remains some uncertainty on the relationship between the present observation of the hierarchical organization of chromosome fibres and those of *in situ* natural structures for the following reasons: ① The surface tention may stretch some of the chromosomes from their natural state; ② Ionic environment at the preparation may modify the extent of packing tightness of nucleosomes into superbeads also from their natural state.

Neverthless, it should be emphasized that the current technique used in this study is capable of observing wide variety of hierarchical organization of chromosome fibers neither fixed nor stained in a hydrated condition with the highest resolution of 10 nm. It has been discussed in detail elsewhere (Shinohara *et al.*, in press) that X-ray contact microscopy is applicable to image hydrated biological specimens at the resolution of 10 nm circumventing the possible factors of artefacts such as radiation damage, thermal diffusion, thermal expansion, temparature increase and the effect of free radicals produced in water.

In summary, we have shown that the direct imaging of wide variety of hierarchical organization in hydrated chromosome fibres is now possible with X-ray microscopy with the resolution up to 10 nm. This accomplishment will introduce a new form of high resolution imaging to biology. By accumulating data from images of condensed and decondensed chromatin fibre*s in sit*u in their natural state, much useful information can be obtained on the organization and on the reguration of the function of chromosomes. In addition, in the future, the use of coherent X-ray from pulsed X-ray lasers and X-ray holograpphic techniques will provide three dimensional images, from which detailed information on the higher configuration of nucleosomes can be obtained.

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FIGURE LEGENDS

Fig. 1. Experimental assembly of a simple hydrated chamber. The wet specimens isolated on a surface of distilled water were mounted on a layer of PMMA supported on a silicon wafer (a). The wet specimens were then covered with a SiN window (b) and press-fitted with a torque gauge (c). This wet assembly was then sea·ed with two pieces of adhesive tape, together with a piece of a wetted filter paper (d.).

Fig. 2. Spectrum of the X-ray flux emitted from the laser produced plasma from Au target. The dotted line show the X-ray flux transmitted through the SiN window.

Fig. 3. An X-ray image of a condensed chromosome fibre (a) and enlarged images of three specific regions identified by framed areas (b to d): b, a cluster of superbeads about 30 nm in diameter (white arrow) ; c, a polynucleosome filament in a series of nucleosomes (thin white arrow); and d, a 30 nm fibre constructed with a polynucleosome filament. Bars represent 1 μm (a) and 100 nm (b-d), respectively.

Fig. 4. Another example of a condensed chromosome fibre (a) and an enlarged image of a framed area (b): Polynucleosome filaments (thin white arrow) and a 30 nm fiber (short and thick white arrow) with tightly packed nucleosomes are evident. Bars represent 1 μm (a) and 100 nm (b), respectively.

Fig. 5. A partially decondensed chromosome fibre (a) and enlarged images of the framed areas (b to f): b, a higher order fibre (thick white arrow) with the size of 60-100 nm in diameter constructed with a helically coiling fibre; c, a knobby 30 nm fibre (small and thick white arrow) made up with superbeads ; d, a polynucleosome filament (thin white arrow); e, a 30 nm fibre with regularly arranged two rows of nucleosomes (white arrow), and f, a series of nucleosome and superbeads. Bars represent 1 μm (a) and 100 nm (b-f), respectively.

Fig. 6. Another example of decondensed chromosome fibre (a) and the enlarged images of the framed areas (b to e): b, a higher order fibre (large white arrow) with a helically coiling 30 nm fibre (small white arrow); c. a cluster of superbeads (white arrow) ; d. a 30 nm fibre with tightly packed superbeads (small white arrow) and a higher order fibre of 60-120 nm in diameter (large and thick white arrow) made up with a coiling 30 nm fibre; and e, superbeads in a moruloid organization. Bars represent 1μm (a) and 100 nm (b-e), respectively.

a.

Specimen

Water

PMMA

Si

b.
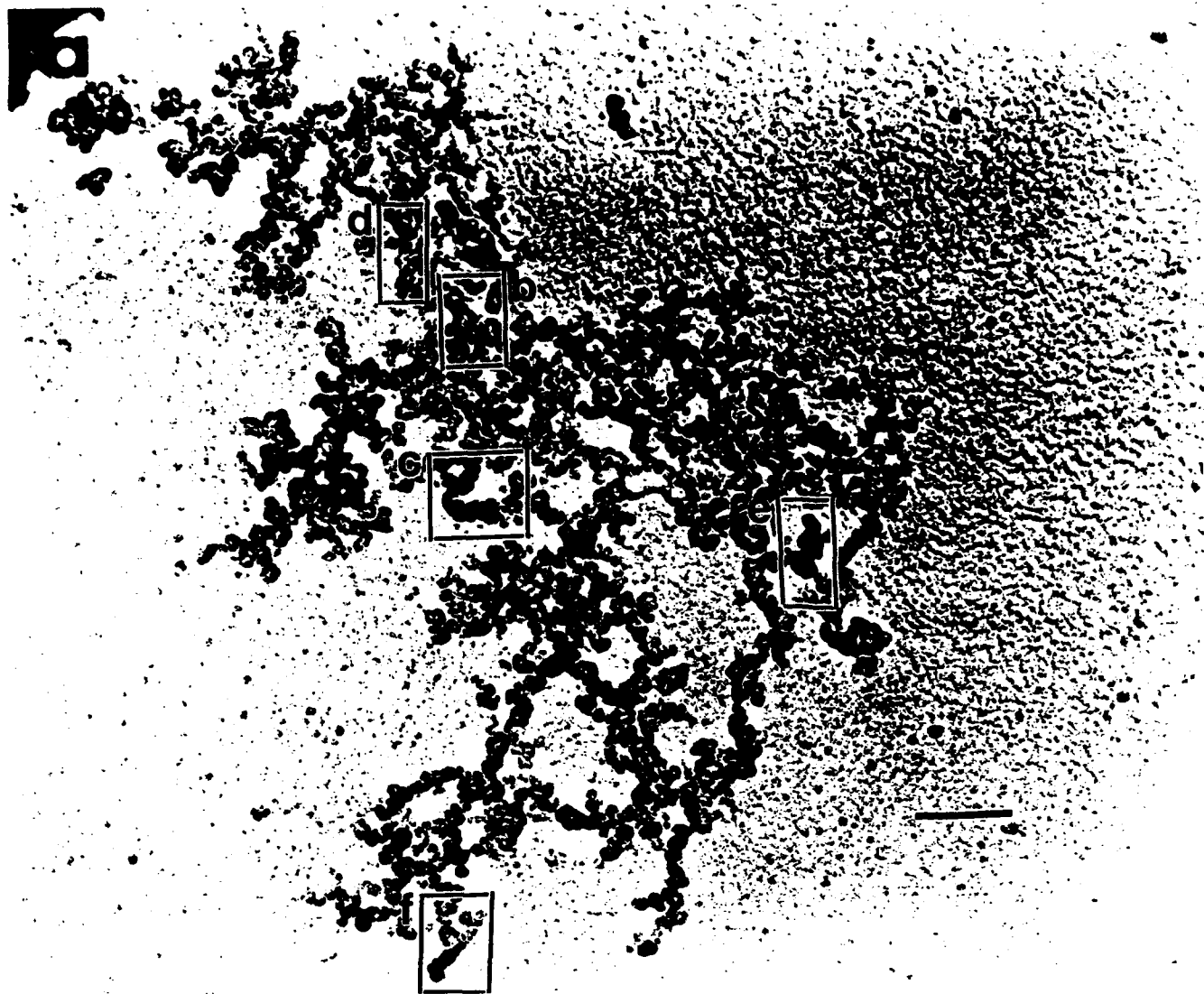
Si

SiN

c.

d.

Sealing tape

Wet filter paper
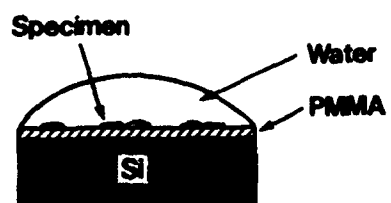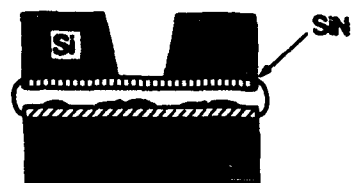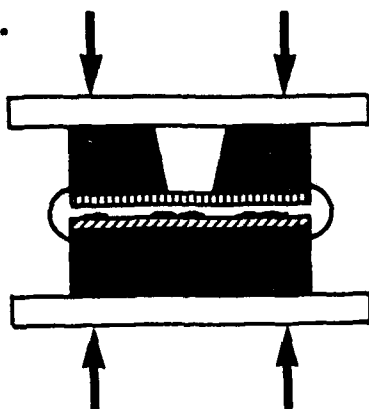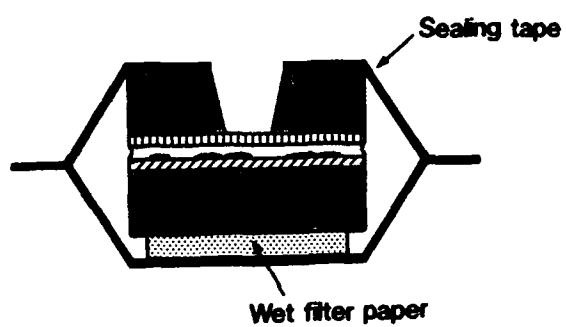
Fig. 1
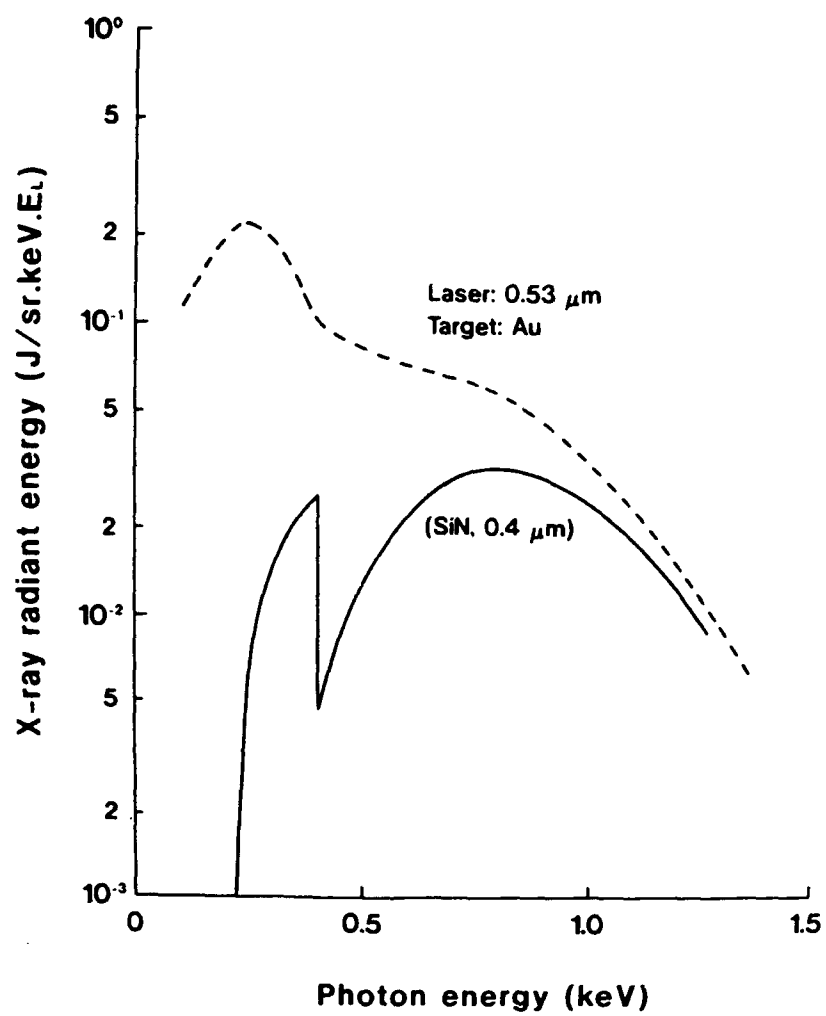
Fig. 2
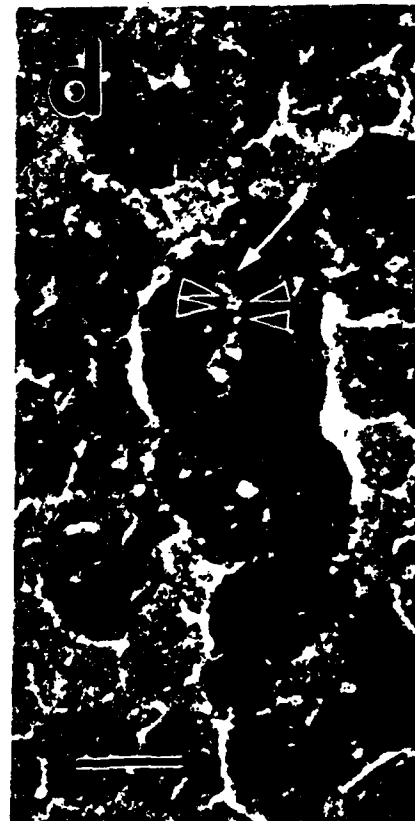
Fig. 3

Fig. 4

Fig. 5

Fig. 5

Fig. 6

Fig. 6